Cell-based models for studying paediatric high-grade gliomas

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“I may not have gone where I intended to go, but I think
I have ended up where I needed to be.”
- Douglas Adams

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Abstract

Brain tumours are the largest group of solid tumours in children and accounts for most cancer-related deaths. Astrocytoma is the largest group, representing almost half of the brain tumours, where high-grade glioma is one of the most devastating forms with very poor prognosis due to lack of efficient treatments. A better understanding of the disease and new treatment options are urgently needed.

In paper I, we assessed the possibility of culturing glioma stem cells (GSC) from primary paediatric high-grade brain tumours, adherently, in serum-free neural stem cell media. Cells maintained proliferative capacity long-term, displayed neural stem cell markers and responded to differentiation cues. Moreover, cell lines initiated tumour growth when orthotopically transplanted into NOG mice.

In paper II, survival of GSC-transplanted NOG mice was monitored, and the histological and molecular features of the developed xenograft tumours were studied. The survival of mice correlated with the survival of the patients. Moreover, the xenograft tumours showed the same growth pattern as the patient tumours with similar genetic and epigenetic alterations as the originating tumour and GSC line.

In paper III, we explored zebrafish as an animal model to study paediatric high-grade gliomas. The tumour-initiating potential and invasive properties were studied. The take-rate of transplanted GSC in

the fish was high, and the cells invaded the surrounding brain tissue of the fish after only a few days.

In this thesis, three pre-clinical models were established which can be used to generate new knowledge and explore new treatment options for paediatric brain tumours.

Keywords: paediatric brain tumour, glioblastoma, glioma stem cell, primary culture, xenograft tumour, zebrafish

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Sammanfattning på svenska

Hjärntumörer är efter leukemi den vanligaste cancerformen som drabbar små barn. Det finns många typer av hjärntumörer och prognosen för en del är god, men ändå står sjukdomen för den största andelen av cancerrelaterade dödsfall hos barn. Överlevnad är beroende av tumörtyp där höggradiga gliom, såsom glioblastom, är en av de diagnoser som uppvisar sämst prognos. Idag finns ingen effektiv behandling, och behovet av nya behandlingsmetoder är stort.

I denna avhandling utvecklades nya pre-kliniska modeller.
I den första studien etablerades ett cellodlingssystem med primära cellkulturer från höggradiga gliom. Tumörceller odlades i serumfritt neuralt stamcellsmedium som adherenta celler. Cellerna uppvisade neurala stamcellsegenskaper i form av asymmetrisk celldelning, stamcellsmarkörer, och potential att bilda hjärnans mogna celltyper (astrocyter, oligodendrocyter och neuroner) samt att bilda tumörer i djurmodeller.

I den andra studien följdes tumörutveckling av transplanterade primära cellkulturer i immunsupprimerade möss. Överlevnaden av mössen korrelerade till överlevnad av patienterna. Xenotransplantaten uppvisade tydliga histologiska och molekylära kännetecken för glioblastom, samt stora molekylära likheter med det ursprungliga tumörmaterialet och cellkulturen.

I den tredje studien undersöktes möjligheten att använda zebrafisk som djurmodell för höggradiga hjärntumörer från barn. De primära cellkulturerna injicerades i hjärnan hos zebrafiskyngel och invasionen av cellerna i hjärnvävnaden följdes.

Sammantaget presenteras tre pre-kliniska, pålitliga och robusta cellbaserade modeller. Dessa modeller kan användas för att generera

kunskap kring glioblastom och bidra till utveckling av nya behandlingsmetoder.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Wenger A, **Larsson S**, Danielsson A, Juul Elbæk K, Kettunen P, Tisell M, Sabel M, Lannering B, Nordborg C, Schepke E and Carén H.

Stem cell cultures derived from pediatric brain tumors accurately model the originating tumors.

Oncotarget, 2017, 8(12):18626-18639. doi:10.18632/oncotarget.14826

II. **Larsson S**, Wenger A, Dosa S, Sabel M, Kling T and Carén H.

Cell line-based xenograft mouse model of paediatric glioma stem cells mirrors the clinical course of the patient.

Carcinogenesis, 2018 Oct 8;39(10):1304-1309. doi:10.1093/carcin/bgy091

III. **Larsson S**, Kettunen P, Carén H.

Invasion of human paediatric high-grade gliomas in zebrafish.

Manuscript

Content

Abbreviation

ACVR1	activin A receptor type 1
ALL	acute lymphocytic leukaemia
ATRT	atypical teratoid/rhabdoid tumour
ATRX	ATRX chromatin remodeler
BBB	blood brain barrier
BET	bromodomain and extra-terminal motif
BMP	bone morphogenetic protein
CDKN2A/B	cyclin-dependent kinase inhibitor 2A/B
CGI	CpG island
CIMP	CpG island methylator phenotype
CNS	central nervous system
CSC	cancer stem cell
CMYC	c-myc proto oncogene protein
DAB	3,3′-Diaminobenzidine
DIPG	diffuse infiltrative pontine glioma
DMP	differentially methylated position
DMEM	Dulbecco's Modified Eagle's Medium
DNMT	DNA methyl transferase
EdU	5-ethynyl-2-deoxyuridine
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial to mesenchymal transition
EZH2	enhancer of zeste homolog 2
FGF	fibroblast growth factor
GBM	glioblastoma multiforme
GFAP	glial fibrillary acidic protein
GSC	glioma stem cell

HAT	histone acetyl transferase
HDAC	histone deacetylase
HGG	high-grade glioma
HIST1H3B	histone cluster 1 H3 family member b
HMT	histone methyl transferase
HRP	horseradish peroxidase
H3F3A	H3 histone family member 3A
IDH	isocitrate dehydrogenase
KDM	lysine demethylase
KDR	kinase insert domain receptor
KIT	KIT proto-oncogene receptor tyrosine kinase
KLF4	kruppel-like factor 4
5mC	5-methylcytosine
MMR	mismatch repair
MYCN	MYCN proto-oncogene, bHLH transcription factor
OCT	optimal cutting temperature
OCT4	octamer-binding transcription factor 4
PBS	phosphate-buffered saline
piRNA	piwi interacting RNA
PDGFR	platelet-derived growth factor receptors
PDGFRA	platelet-derived growth factor receptor alpha polypeptide
PDX	patient-derived xenograft
PDOX	patient-derived orthotopic xenograft
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PNET	primitive neuroectodermal tumour
PRC2	polycomb repressor complex II
PTEN	phosphatase and tensin homolog
PXA	pleomorphic xanthoastrocytoma
RB1	RB transcriptional corepressor 1
RTK	receptor tyrosine kinase
SHH	sonic hedgehog
siRNA	short interfering RNA

SOX2	SRY (sex determining region Y)-box 2
SVZ	subventricular zone
TET	ten-eleven translocation
TMZ	temozolomide
TP53	tumor protein p53
VPA	valproic acid
VEGF	vascular endothelial growth factor
WHO	world health organisation
WNT	wingless integrated
2D	two-dimensional
3D	three-dimensional

Introduction

The word cancer comes from the Greek physician Hippocrates (460-375 BC) who described a disease that he named karkinos because its growth pattern reminded him of a crab or cray fish. Today we use the term cancer, which is the Latin word for karkinos [1]. The disease was mentioned in old writings as early as approximately 3000 BC in the "Edwin Smith Papyrus" [2].

In our body, all cells follow specific rules due to body architecture and life cycles. If cells leave their predetermined life and start a journey on their own, cancer begins. On its way, cells acquire capabilities that are necessary to become tumourigenic. Hanahan and Weinberg first proposed six so-called hallmarks of cancer; sustaining proliferative signals, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and resisting cell death [3]. With the fast and progressive research in the cancer field, an updated version of additional hallmarks was presented including; genome instability and mutation, deregulating cellular energetics, avoiding immune destruction and tumour promoting inflammation [4].

Childhood cancer

Childhood cancer is rare. According to a report from the Swedish Childhood Cancer registry, 16.0 per 100 000 children <15 years of age are diagnosed with a primary cancer disease each year in Sweden [5]. Under the age of ten, the most common diagnoses are leukaemias, representing 30% of cases, and central nervous system (CNS) tumours, 28% of cases.

The 5-year survival for all types of cancer diseases have increased greatly. Most improvements have been for acute lymphocytic leukaemia (ALL) where the disease was fatal in the 1970s, but show a 5-year survival of almost 90% today [5]. Even though survival rates differ considerably between cancer types, the increased survival for specific groups encourage and inspire more research for all types of tumours to gain knowledge for improved treatment options in the future.

Childhood brain tumours

Within solid tumours, CNS tumours are the most common type and account for the highest number of cancer-related deaths in children <15 years of age [5, 6]. The incidence is slightly higher in boys (sex distribution ratio males/females = 1.10). There is no typical age peak for CNS tumours in total, but some tumour types show specific age distributions [5].

There are many different types of childhood brain tumours and this paragraph will describe the most prevalent ones. Gliomas are the most common type of brain tumours. They originate from neuroepithelial tissue, the supporting tissue of the brain [7, 8]. The prognosis is highly dependent on the grade of the glioma. Within gliomas, astrocytomas are the largest group accounting for approximately 45% of all brain tumours. Astrocytoma is subdivided into four grades; low grade I and II (~31% of all brain tumours) and high grade III and IV (~6% of all brain tumours), where the 5-year survival rates differ largely between 90% and 30%). Other types of gliomas in children are ependymomas, originating from ependymal cells that line the ventricles (10.5%); oligodendrogliomas, originating from oligodendrocytes (2.5%) and brainstem gliomas originating from any neuroepithelial cell located in the brainstem (2.6%) [7].

The second largest group of brain tumours is embryonal tumours (18.8%). The most common type is medulloblastoma, originating from cells in the

cerebellum, representing 14.8% of all brain tumours. Atypical teratoid/rhabdoid tumour (ATRT) is a less common type of embryonal tumour (0.3%) [7]. The diagnosis primitive neuroectodermal tumour (PNET) was removed from the last world health organisation (WHO) classification as it was shown that these tumours represent other established diagnoses [8, 9].

Paediatric high-grade glioma

Glioblastoma multiforme (GBM) is a grade IV astrocytoma and one of the most devastating forms of brain tumours. Unlike adult GBM, which is the most frequent malignant brain tumour, paediatric GBM is rare with an incidence of only 2.7% [10], though both share the same poor 2-year survival of only 15-30% and 20-32% respectively [7, 10-13]. Other high-grade gliomas (HGG) are anaplastic astrocytoma and diffuse infiltrative pontine glioma (DIPG) [8].

The advancements made in molecular profiling of HGG during the last decade have unravelled features not detected by histological analysis. With the help of genetic and epigenetic features, the paediatric HGG tumours can be divided into six groups; K27, G34, IDH (isocitrate dehydrogenase), RTK (receptor tyrosine kinase) I, Mesenchymal and PXA (pleomorphic xanthoastrocytoma)-like tumours [14]. The groups are based on DNA methylation data, age of onset, tumour location, gene expression, oncogenic drivers, and survival time. The molecular signature is a prognostic factor, where K27 tumours show shorter survival time [14-16].

Genetic aberrations and mutations are less common in paediatric HGG than in adult brain tumours. The most common copy number alterations (CNA) are amplifications including the genes platelet-derived growth factor receptor alpha polypeptide *(PDGFRA),* KIT proto-oncogene receptor tyrosine kinase *(KIT),* kinase insert domain receptor *(KDR),* MYCN proto-oncogene, bHLH transcription factor *(MYCN)* and

epidermal growth factor receptor (*EGFR)*. Deletions most commonly occur in cyclin dependent kinase inhibitor 2A *(CDKN2A)*, phosphatase and tensin homolog *(PTEN)* and RB transcriptional corepressor 1 *(RB1).* Somatic mutations are frequently observed in the genes H3 histone family member 3A *(H3F3A)*, tumour protein p53 *(TP53)*, ATRX chromatin remodeller *(ATRX)*, activin A receptor type 1 *(ACVR1)* and histone cluster 1 H3 family member b *(HIST1H3B)* [17].

Survival from GBM in children is poor and treatment strategies that prolong survival in adult GBM show no effect in children [18]. The understanding that adult and paediatric GBM are molecularly very different, despite sharing the same name, has led to new ideas and altered treatment strategies [19].

In a phase 2 clinical trial, treatment of paediatric HGG patients with additional adjuvant treatment (temozolomide (TMZ) and lomustine) after standard care (radiation and TMZ) showed an increased survival time compared to patients treated with standard care only [20]. In a more recent study, the varying response to chemotherapies for the whole group of paediatric HGG emphasised the need and importance to direct treatment within this group [21]. Molecular profiling, in addition to current diagnostic methods, can facilitate this work. The gain of new information regarding molecular status of tumours should be considered as additional input for pathologists evaluating tumour samples to enable accurate diagnosis. During the last years, methylation-based algorithms to classify brain tumours have been published, which identify diagnostic entities and subgroups [9, 22-24]. The molecular profile of tumours not only helps in diagnosing tumours, but also provides additional prognostic information and enables targeted treatment designs.

Valproic acid (VPA), an epigenetic drug, has been used as continued treatment after complete resection, radiation and intensive chemotherapy [25]. In addition, the identification of deregulated components of the

epigenetic machinery has led to the discovery of new druggable candidates [26]. Enhancer of zeste homolog 2 (EZH2) [27], DNA methyl transferase (DNMT) I [28], histone deacetylase (HDAC) [29] and bromodomain and extra-terminal motif (BET) [30-32] are some of the potential candidates that can move the field forward.

Epigenetics

The term Epigenetics, coined by Conrad H. Waddington in 1942, comes from the Greek word 'epi' referring to over, above, around and 'genetic' referring to the science of heredity [33]. All cells in our body carry the same genetic code, but still exhibit different phenotypes. This is possible due to epigenetics. The definition made by Michael K. Skinner "molecular factors and processes around DNA that are mitotically stable and regulate genome activity independent of DNA sequence" in 2010 summarises the concept well [34]. Epigenetics alter the transcription and translation of genes in the cell. Upon differentiation, the cell is "locked" into a certain path by epigenetic mechanisms and destined to become a specific cell type.

Epigenetic machinery

Epigenetics works through three mechanisms; DNA methylation, histone modifications and non-coding RNA (Figure 1) [35].

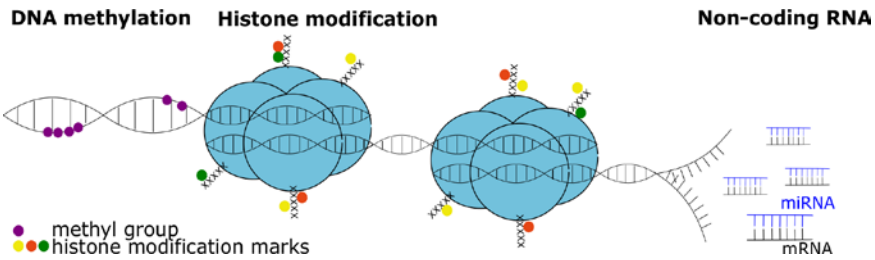

Figure 1: The epigenetic machinery.

DNA methylation

DNA methylation was first described by Holliday and Pugh in the 1970s, and was demonstrated to be involved in gene regulation in the beginning of the 1980s [36, 37]. Methylation has since then been shown to play an important role in biological processes such as development, ageing, genomic imprinting and X-chromosome inactivation [38].

The DNA strand contains four nucleotides; guanine (G), cytosine (C), adenine (A) and thymine (T) [39]. Methylation most often occurs on the 5' carbon residue of cytosine coupled to a guanine and are referred to as CpG sites. Throughout the genome, these sites are heavily methylated [40]. The methylation process is carried out by DNMT transferring a methyl group from S-adenosyl methionine. DNMT1 is involved in sustaining methylation after mitosis, and DNMT3a and b are responsible for *de novo* methylation of DNA.

Regions on the DNA strand that contain a high number of cytosines followed by guanines are called CpG islands (CGI). These are enriched in promoter regions and enhancer regions upstream of genes. CGI are predominantly unmethylated [41].

Epigenetic modifications are reversible. Methyl groups can be removed from cytosines through passive or active processes. A decrease in DNMT1 results in a reduction of methylated cytosines in a passive way via mitosis. The active process can occur in at least three different multiple step ways, involving hydroxylation of 5-methylcytosine (5mC) by the ten-eleven translocation (TET) family enzymes or deamination and finally replacement of the resulting cytosine intermediates by base excision repair (BER) pathways to naked cytosines [42].

Histone modifications

DNA is wrapped around nucleosomes, which are octamer protein complexes consisting of duplicates of the histone proteins H2A, H2B, H3 and H4. Each histone has a residue that can be modified by different epigenetic marks (chemical groups), for example methylation, acetylation, ubiquitination and phosphorylation.

The modifications are processed by epigenetic writers; enzymes such as histone methyltransferase (HMT) and histone acetyl transferase (HAT), which add an epigenetic mark, epigenetic erasers; enzymes which remove the mark such as histone deacetylase (HDAC) and lysine demethylase (KDM) and executed by epigenetic readers; chromodomain-, tudor domain- and bromodomain proteins which help interpret the modifications. Together these three types of proteins regulate the DNA in terms of active/repressed transcription, DNA repair and DNA replication [43, 44].

Different modifications constitute specific histone codes; acetylation of lysine is for example associated with gene activation. Methylation of lysine, on the other hand, has different effects depending on the residue that is methylated. Methylated K4 at histone 4 is associated with active transcription, while methylated K27 and K9 at histone 3 and K20 at histone 4 are associated with transcriptional repression. Together these histone modifications form a flexible, but very precise DNA packaging [45].

Non-coding RNA

Non-coding RNA is RNA that is not translated into protein. There are short non-coding RNA, such as microRNA, short interfering RNA (siRNA) and piwi interacting RNA (piRNA), and long non-coding RNA. Both groups are involved in silencing gene expression by post-transcriptional modification [46].

Epigenetic alterations in cancer

Initiation and progression of cancer are associated with epigenetic alterations of the genome. In cancer, the DNA becomes globally hypomethylated, mostly due to loss of methylated Cs in repetitive regions and demethylation of coding regions and introns [47, 48].

Three proposed events, explaining the unstable signature of cancerous cells due to hypomethylation, are induction of oncogene activation, generation of chromosomal instability via demethylation of repetitive elements, and loss of imprinting [47]. In addition, hypermethylation of CGI close to promotor regions of tumour suppressor genes is also common in tumour cells [48].

Specific histone modifications play a crucial role in affecting chromatin structure and gene transcription. These alterations are commonly associated with DNA hypermethylation of tumour suppressor genes [49]. The plasticity of CSC and their epigenetic marks in the brain is an important issue. Previous studies on leukaemia show that terminal differentiation of tumour cells may be used to stop tumour growth [50]. Even though early data suggested that differentiation-based therapy was feasible also for GBM [51], Carén et al later showed that differentiation of glioma stem cells (GSCs) does not lead to terminal cell cycle exit [52]. GSC responded rapidly, within days, to astrocyte differentiation cues using bone morphogenetic protein (BMP) 4 with changed morphology, expression of astrocyte markers and stopped proliferation, but this was only temporary. Re-exposure to growth factors to both short- and long-term differentiated cells reactivated proliferation and entrance into the cell cycle. As DNA methylation changes does not occur as rapid in GSCs as in other more short-lived cells such as leukocytes, the epigenetic machinery of the more long-lived cells of the brain needs to be considered in future studies.

Epigenetic alterations in paediatric high-grade gliomas

Genes coding for histone proteins are highly conserved and stable. However, somatic mutations do occur in these genes and are associated with cancer. The genes *H3F3A* and *HIST1H3B/C* encode for proteins on histone 3 and mutations are associated with amino acids substitutions on the histone residues [53-55]. The mutations are frequent in paediatric HGG and referred to as H3K27M and H3G34R/V [55, 56]. Mutations are location-dependent; H3K27M is found in the midline of the brain and H3G34R/V is found exclusively in the hemispheres [57]. H3K27M leads to global hypomethylation when the amino acid lysine is replaced by a methionine. The subunit EZH2 of the polycomb repressor complex II (PRC2) normally mediates the methylation of lysine, but when H3K27 is mutated, the methylation is decreased due to less affinity for methionine by EZH2. This leads to de-repression of genes, which could be oncogenic. H3K27M in HGG is associated with shorter survival time [58].

The other histone mutation, H3G34R/V, also results in global hypomethylation. The mutation results in the replacement of guanine by arginine or valine, and decreases methylation at the nearby K36 position on the histone residue leading to increased transcription of, for example, the oncogene *MYCN*. Moreover, mutations in *SETD2 (*methyl transferase) have been correlated to a decrease in the DNA mismatch repair (MMR) system [59, 60]. Mutations in the *ATRX* gene (involved in telomere lengthening) have been identified in one third of the G34R/V group of paediatric glioma patients [57, 60].

Unlike genetic mutations, epigenetic alterations are reversible. The possibility to reverse tumour-driving properties could be employed for developing novel treatment and targeted therapies. Targeting one or many epigenetic signature marks in pHGG have been tested during the last years as described below.

DNMT inhibitors

The DNMT inhibitor decitabine has previously been described to induce differentiation and decrease proliferation of glioma cells [61]. In another study, Rajendran et al showed increased levels of all three *DNMT*s in glioma cells. When treated with the DNMT inhibitor 5-azacytidine, the expression levels of *DNMT1* and *3b* levels were decreased and an enhanced expression of tumour suppressor genes such as *PTEN* was shown [62]. Yet another study showed that downregulation of *DNMT1* leads to enhanced chemosensitivity [63]. These results suggest that DNMT inhibitors might be used in cancer therapy. No clinical study on DNMT inhibitors has been presented in paediatric GBM, but a study on medulloblastoma showed promising results with a combination of the DNMT1 inhibitor decitabine and the HDAC inhibitor vorinostat [64].

Histone modification inhibitors

The histone modifier vorinostat, an FDA approved drug, is the most tested HDAC inhibitor for brain tumours. It has been evaluated individually and in combination with chemotherapy or radiation on primary and recurrent paediatric HGG in several clinical trials [29, 65-67], but no significant response has been reported. Another HDAC inhibitor, VPA, has also been tested for toxicity and anti-tumour activity in a clinical trial of paediatric solid tumours. Two out of the 26 enrolled patients showed response (GBM) and minor response (brainstem glioma) to the treatment [68]. Other studies on paediatric HGG and GBM have been presented earlier [69, 70].

Another histone modifier is the JMJD3 (H3K27 demethylase) inhibitor GSKJ4. Promising results have been reported *in vitro*, and in *in vivo* studies of xenotransplanted mice where tumour growth reduction was shown [71]. Another epigenetic drug targeting the methylation of histone tails is a small molecule inhibiting EZH2. In DIPG, H3K27M cells rely on PRC2 for proliferation and EZH2 is therefore a potential target in these tumours [27].

BET inhibitors

The epigenetic readers, including BET, are also possible targets for improving therapy. In adult GBM, the BET inhibitor dBET6 represses the proliferation and self-renewal of GBM cells [32]. Treatment with a BET inhibitor in combination with an EZH2 inhibitor in mouse DIPG cells exhibited better response and inhibited tumour growth both *in vivo* and *in vitro* when drugs were used in combination rather than individually [72]. Targeting multiple pathways or modifications in heterogeneous GBM tumours will most likely be the key to improve therapeutic response.

The origin of cancer

In 1956, Otto Warburg presented his explanation to why cells become tumour cells in the article "on the origin of cancer cells" [73]. This included metabolic changes in a cell due to fermentation and respiration alterations after chemical stress [73]. His theory is highly relevant even today [4].

Two different theories on the origin of cancer have been proposed. 1) The stochastic model, where progenitors or mature cells in a tumour environmental niche gain genetic or epigenetic lesions. This model leads to a tumour with multiple sub-clones where the best-fitted tumour cell drives the tumour growth [74, 75]. 2) The cancer stem cell (CSC) model, where one cell initiates tumour growth and gives rise to further mature cells in an hierarchical organisation which resembles that of stem cells (Figure 2). Sub-clones can form further down in more differentiated stages of tumour cells. The CSC theory have been proposed in for example leukaemia [76], brain tumours [77] and breast tumours [78].

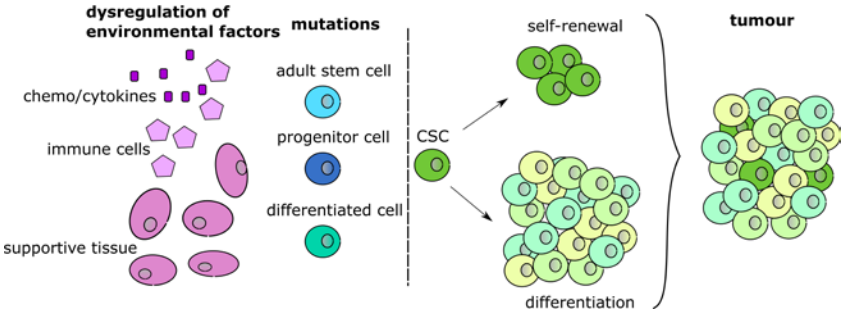

Figure 2: The cancer stem cell theory.

Stem cells

All cells in our body originate from that first cell, in the beginning of everything. This cell is a totipotent embryonic stem cell, with the capacity for self-renewal, the potential to differentiate into all germ layers (mesoderm, endoderm and ectoderm) and the capacity to form necessary embryonic tissue and parts of the placenta. The totipotency is lost after a few cell divisions, and a pluripotent embryonic stem cell remains. This stem cell maintains the capacity of self-renewal and potential to differentiate to all germ layers [79].

Pluripotent stem cells are of great interest for organ regeneration and transplantation medicine. Reprogramming of somatic cells has been performed since John Gurdon entered the stage using nuclear reprogramming to make a frog from an egg and a somatic cell in 1960 [80]. Over time, reprogramming has evolved through different types of delivery methods such as PiggyBac, lenti/adeno virus, episomal transfection, Sendai virus, small molecules and mRNA technology. In 2006, Shinya Yamanaka first published the protocol for making induced pluripotent stem (iPS) cells by adding only four transcription factors; octamer-binding transcription factor 4 (OCT4), Kruppel-like factor 4 (KLF4), SRY (sex determining region Y)-box 2 (SOX2) and c-myc proto oncogene protein

(C-MYC) to mice fibroblasts [81]. This has subsequently been successfully performed also using human somatic cells [82]. Exogenous expression of pluripotency transcription factors force the cell to start transcribing the factors endogenously. This push towards pluripotency makes the cell undergo epigenetic changes which drives the cell back to an immature state close to that of embryonic stem cells [83].

Adult stems cells are multipotent cells with a lineage-specific differentiation path. They are responsible for maintaining homeostasis and repairing cells upon injury or cell death, and are rare in the body. Adult stem cells are characterised by the capacity to self-generate throughout the lifetime of the organ. They should give rise to differentiated cells with a mature phenotype that constitute all cell types of the specific tissue. They should also integrate into the tissue with appropriate functions of the tissue it resides in [84].

The definition of adult stem cells is by many researchers based on only two characteristics; morphological appearance and the potential to differentiate. One should be aware that adult stem cells are almost impossible to distinguish from progenitor cells in the body. Progenitor cells are partially differentiated cells that give rise to more differentiated cells, but do not possess self-generating ability. To claim the existence of adult stem cells, the ability of self-generation throughout life needs to be fulfilled [84].

The origin of adult stem cells is unknown. One proposed theory is that partially differentiated cells with self-renewal capacity are set aside early in the developing process and retained in the organs throughout life [84]. Adult stem cells have been identified in the human brain, in the subventricular zone (SVZ) and in hippocampus [85, 86]

… and cancer stem cells

Solid tumours are accumulations of cells with uncontrolled proliferation and vascular growth. The tumour constitutes cells in different maturation stages, presenting various cell surface markers, genetic and/or epigenetic changes and growth rates. Moreover they respond to therapy differently [87].

Historically, the theory of dormant cells with stemness characteristics that give rice to cancer was proposed by the German pathologist Rudolf Virchow as early as 1855 [88]. It was not until 1994 the modern theory of CSCs was proposed, explaining the role of cancer cells in leukaemia, sharing characteristics with normal stem cells [89]. In 2003, CSCs were identified in human brain tumours [90] and breast cancer [91].

The American Association of Cancer Research has defined a CSC as a cell within a tumour with the capacity of self-renewal and that gives rise to the heterogeneous lineages of cancer cells that constitute the tumour [92]. When transplanted into mice, human CSCs have been shown to not only grow, but also to form multiple cell layer tumours similar to its originating tumour [91]. A tumour cell that gains stem cell properties alters pathways necessary for stemness, such as the wingless integrated (Wnt)/β-catenin, sonic hedgehog (SHH), Notch and telomerase reactivation pathways. While altering these pathways, the tumour cell undergoes a transition from epithelial cell structure to mesenchymal cell structure known as EMT (epithelial to mesenchymal transition). During this transition, epithelial polarity is lost, cell morphology changes and E-cadherin is down-regulated while N-cadherin is upregulated. EMT can also push the cell into a more quiescent state [93].

The term CSC is debated and in the literature, these cells are also called cancer-initiating cells, cancer stem-like cells or cancer progenitor cells. It

is important to note that CSCs do possess stem cell properties, but are not necessarily stem cells.

Cancer stem cells in HGG

CSCs in GBM were first reported by Singh in 2004, when he showed that not all tumour cells were able to form new tumours *in vivo*. In this study, he demonstrated that the cells that initiated tumours were exclusively cells expressing the neural stem cell marker CD133 [90]. Other studies have since then, in controversy with Singh, shown that different CSC populations exist with both CD133+ and CD133- cells as well as only CD133- cells. Tumours driven by CD133- CSCs were found to be less malignant [94]. If the coexistence of CD133+ and CD133- CSCs in GBM occurs, and should be considered a hallmark of the disease, needs to be further investigated [94]. The coexistence of different CSCs in GBM has been demonstrated in other studies, strengthening this theory [95, 96]. Other surface markers such as integrin α6, CD15/SSEA1 and CD44 have also been proposed as CSC-specific in GBM [97, 98].

A study by Suva et al presented four transcription factors; POU3F2, SOX2, SALL2, and OLIG2 that could turn a differentiated GBM cell into a tumour-initiating cell. These proteins have also been reported important for the maintenance of the CSC state [99].

The maintenance of glioma CSCs depends on many factors, for example metabolism, epigenetic/genetic factors, the tumour niche and environmental factors. Levels of hypoxia and oxygenation alter the metabolism in the cell. When cells are under hypoxia, migration is stimulated and cell proliferation is decreased [100]. In addition, EZH2, which methylates H3K27 and alters the chromosomal structure of the DNA, has also been proposed to be important for maintaining the CSC state [101].

The origin of CSCs is not clear. In a study by Jeong Ho Lee et al, the authors proposed that the origin of CSCs is adult stem cells migrating from the SVZ. In this study, patient material from three different zones in the brain in relation to the GBM tissue was collected and profiled. They showed that approximately 50% of the 55 patients with GBM had cells in the SVZ containing low-level driver mutations for GBM [77].

Cause of relapse

When not all cells in the tumour respond to therapy, tumours can relapse (Figure 3). The most common treatment following diagnosis is surgery, radiation and chemotherapy. Lately, more advanced and sophisticated tumour-specific pre-clinical treatments have been introduced using epigenetic drugs [61, 64].

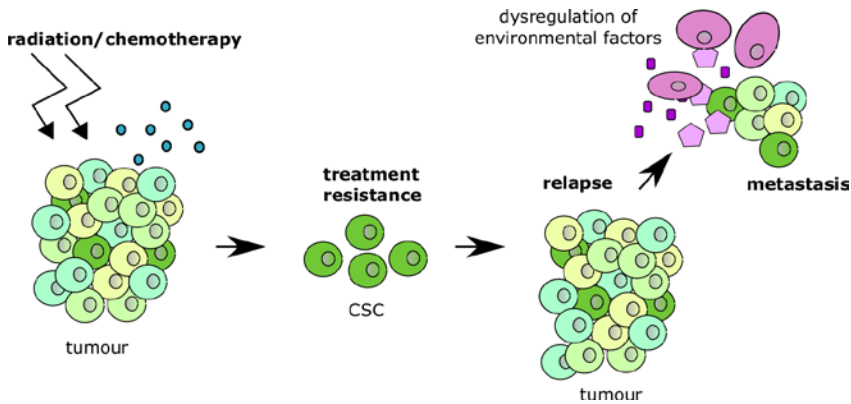

Figure 3: CSC resistance to treatment.

Within tumours, cells respond to therapy in different ways. Patients with brain tumours that display CD133 have a worse 5-year survival than those with CD133 negative tumours [102, 103]. Moreover, stem cell properties have been proposed to be the main reason for therapy resistance. CSCs possess intrinsic factors that make them less susceptible to treatment than more differentiated stages of cells in the tumour. Intrinsic factors that contribute to drug resistance are:

- **stemness induction**; alteration of stem cell pathways and EMT [93].
- **drug export**; efflux of toxic elements in the cell via ATP-binding cassette transporters highly expressed on the cell surface of the CSCs [104].
- **high cell survival**; inactivation by, for example mutations, of cell cycle regulating genes and apoptotic genes preventing CSCs from entering apoptosis [105].
- **hypoxia**; in a hypoxic microenvironment, CSCs activate hypoxia-inducible factor 1-alpha (HIF1α), which leads to EMT and alteration of stemness pathways [106].
- **quiescence**; at a quiescent state, CSCs stop dividing and consequently escape treatment that targets fast dividing cells [107].

Because of these intrinsic factors, CSCs carry endogenous resistance mechanisms against radiotherapy and chemotherapy in a larger extent than non-CSCs (tumour bulk cells) [103]. In addition, resistance is also partly due to the slower proliferation rate that CSCs possess since current therapy targets fast dividing cells [108]. Finding treatment that target CSCs is thus important for improving therapeutic efficacy.

Model systems

Thomas Henry Huxley, an English biologist specialised in comparative anatomy, introduced the principle “We are unlikely to ever know everything about every organism. Therefore, we should agree on some convenient organism(s) to study in great depth, so that we can use the experience of the past (in that organism) to build on in the future. This will lead to a body of knowledge in that 'model system' that allows us to design appropriate studies of non-model systems to answer important questions about their biology” as Kunkel concluded it [109].

In the field of medical research, moral and ethical questions should always be on the agenda. In "The Principles of Humane Experimental Technique" William Russel and Rex Burch coined the three R´s: **reduction**, **refinement and replacement** [110]. Every researcher should strive to fulfil these important words in their research.

In vitro models

Human cells can be grown *in vitro*; maintained in cell culture media and stored in an incubator with optimal conditions of CO_2, pH and temperature. Cells can be cultured in many ways; most commonly as two-dimensional (2D), or in three-dimensional (3D) structures. Cells can also be grown in co-cultures, e.g. different cell types mixed to create better culture conditions.

The first human cell line was derived from a cervical cancer biopsy in 1951 by George Otto Gey, a biologist at John Hopkins University. The tumour cells came from a woman named Henrietta Lacks and were simply named HeLa from the first two letters of her first and last name. The HeLa cells grow immortally and have been used by thousands of researchers since it was established [111]. Today, almost any cell type can be purchased as a well-characterised cell line. Cell lines established from patient material, that have undergone only a few doublings are called primary cell lines and have been shown to be more representative as *in vitro* models since they possess more similarities with the original tumours than non-primary cell lines [112, 113].

Culturing HGG cells

HGG cell lines and primary cultures have been used in medical research to characterise and gain knowledge about specific features and therapeutic responses. Many HGG cell lines used today are derived from adult GBM.

The benefit of primary adult cell lines as an *in vitro* model instead of non-primary cell lines is that the primary cells reflect the patient tumour better, which has been shown by several groups [114, 115]. Moreover, focusing on only CSCs in the cultures demand serum-free culture conditions since serum alters the pheno- and genotype of stem cell cultures [116].

Bax et al described the phenotypic and molecular differences between paediatric and adult GBM highlighting the need for paediatric models since many cell lines are established from adult tumours [117]. The first primary cell model of paediatric GBM was established during the 1980s. Cell cultures from many paediatric brain tumours have since been established, mostly for medulloblastoma and ATRT. These cell cultures are serum-based and the cells grow as spheres [118]. It was not until recently the first serum-free CSC cultures from high-grade paediatric brain tumours were presented grown as spheres and adherent cells [119]. However, none of the established cell lines was GBM.

All model systems have disadvantages; the lack of environmental stimuli in cell cultures is one. Co-cultures could partly overcome this disadvantage. In addition, cells do not grow in 2D other than in cultures, which makes them sensitive and more vulnerable to exogenous factors. 3D cultures could act as a protective state for the cells. Moreover, translating drug experiments and concentrations can be hard since tumour cells *in vitro* are fully exposed to the drugs in contrast to tumour cells in the body surrounded by tissue. Still, *in vitro* cultures enable high-throughput screening with the potential of discovering new active substances. One should however bear in mind that many drugs that have shown great potential *in vitro* have later shown to be ineffective *in vivo* [120]. This again emphasises the need of several models.

In vitro models can be used to gain knowledge and experimental results that can be transferred to the next level, *in vivo* models, with **replacement**, **reduction** and **refinement** in mind.

In vivo models

The most common and well-known vertebrates in research are mice, xenopus, chicken and zebrafish. A good model organism should be easy to handle, inexpensive to maintain, have a short life cycle, be possible to genetically modify and have the potential to deliver economically important results [121]. The possibility to study human tumour cells in a more natural environment is of great relevance in the translational context compared to cells grown in a petri dish.

Mouse (mus musculus)

The most common and well-known vertebrate used in brain tumour research is immunodeficient mice [122]. Mice fulfil all the criteria for a good animal model as mentioned above. Patient-derived xenograft (PDX) models of paediatric high-grade brain tumours have been presented. In a study by Houghton et al, primary high-grade brain tumour tissue was injected subcutaneously in immune-suppressed mice [123]. The environmental impact on the tumour cells should ideally be studied in the right context, therefore orthotopic models could be beneficial. Patient-derived orthotopic xenograft (PDOX) injection of primary medulloblastoma tissue and DIPG sphere cultures have been shown to grow and initiate tumour formation in immune-incompetent mice [124, 125]. Most recently, Brabets et al presented a PDOX model based on injection of multiple entities of paediatric brain tumours, including GBM, where tumour tissue and cultured spheres were injected [126].

Zebrafish (danio rerio)

The zebrafish is an animal model with great potential and a feasible option in cancer research. It has been used in developmental, disease, toxicology, inflammation and cancer-related studies and transgenic animal models have been used to study development, inflammation, blood brain barrier (BBB) and brain tumours [127-135].

Whole genome sequencing of zebrafish has shown similarities with the human genome; we are not as different as one might have hoped. Approximately 71% of the human genes have an orthologue in zebrafish. Of the disease-related genes in human, 82% have at least one orthologue in zebrafish [136].

Zebrafish embryos are see-through and maintained in a petri dish making them easy to monitor compared to mice foetus. Zebrafish also lack a developed immune system in early larval stage thus making them suitable for xenotransplantation. In addition, zebrafish enables a high-throughput setting compared to mice since they produce many offspring, are easier to inject and treat, and require less space.

Zebrafish models of GBM

Studying human tumour cells in zebrafish **reduces** and **replaces** the number of higher vertebrates such as mammals used in a study. It also **refines** the experiment as target mechanisms can be understood or drug candidates identified prior to the use of mice. Zebrafish experiments could therefore act as a step between cell culture and mice experiments.

Previous zebrafish xenograft glioma models have been established by injecting rat glioma and human glioma U87 cells into the yolk-sac (the source of nutrition for the embryo) of the embryo, studying the migration of tumour cells [137-139]. However, the importance of studying cells in their natural microenvironment, the brain, was emphasised by Lal et al. They showed that glioma cells injected into the yolk of the embryo resulted in an absence of invasion into the surrounding tissue, questioning the relevance of that model [140]. Orthotopic models have therefore been developed to meet the demands of biological relevance. In a study by Welker et al, adult primary human GBM spheres survived when orthotopically transplanted into the fish brain and later responded to treatment [141].

Eden et al presented a different approach using adult zebrafish orthotopically transplanted with murine GBM cells [142]. This approach could be more useful for studying adult GBM, since the host mimic the adult setting better. In this study, the fish needed to be immunosuppressed since the adult fish is immune competent.

In a recent study, a new model was presented [143]. Human adult GBM cell lines and primary GBM cells were injected into the blastula of the embryos. The glioma cells subsequently migrated to the brain of the 24h old embryo. This model provides a quicker experimental procedure since the larvae does not have to be anesthetized or embedded prior to injection.

How suitable are zebrafish for testing human drugs?

Zebrafish as an animal model in drug research offers technical and biological advantages. However, are fish suitable for testing human drugs? Do they provide clinical relevance? Humans and zebrafish are phylogenetically further apart than humans and mice and it is important to understand these differences when evaluating the capabilities and limitations of this model. As mentioned, zebrafish harbour an orthologue counterpart to more than 80% of human disease-related genes. Ten of the most prescribed human drugs have target genes with sequences that match with more than 50%. This might not strike as similar, but the active binding site of the protein is in the most conserved parts of the gene sequence, which makes it functionally more similar than at a first glance [144].

The physiology of organs in fish matches the human and carry out the same functions. For example, the zebrafish pancreas comprises all the same cell types as the human pancreas and drugs that alter the glucose homeostasis show the same effect in fish as in humans [145]. Likewise, the haematopoietic system is built up by the same cell types [146] showing conserved haematopoietic pathways. This results in a similar cellular response to drugs affecting haematopoiesis and anaemia in humans and

zebrafish [147]. Interestingly, key factors in these pathways and processes were first discovered in zebrafish [148]. Many cardiovascular drugs have been tested in zebrafish with similar response as in humans [149]. Even more interesting is that zebrafish share more similarities with the human cardiac electrophysiology than rodents do to humans.

Not only do small molecules affect cells in the same way in zebrafish as they do in humans, there is also evidence for drugs passing through biological boundaries as the BBB and tissue-specific transporters in the same way in zebrafish as in humans [150, 151]. In an orthotopic xenograft study of GBM in zebrafish by Zeng et al, a nitrogen mustard-based DNA cross-linking small chemical, TN-B, was evaluated as a treatment candidate [152]. To test if the molecule could cross the BBB, TN-B was injected simultaneously with doxorubicin (known for not passing the BBB), resulting in doxorubicin remaining in the capillaries of the brain while TN-B crossed and diffused out into the brain. Treatment with TN-B resulted in a decrease of the xenograft tumour [152].

To summarise, the zebrafish offers a suitable and complementary animal model to the mouse models commonly used today. Zebrafish bridges the gap between *in vitro* and higher *in vivo* models while taking **reduction**, **refinement** and **replacement** into account.

The use of *in vitro* and *in vivo* models in pre-clinical studies paves the way to reach novel treatment options (Figure 4). The molecular insight into treatment response in several models increases the knowledge for further testing.

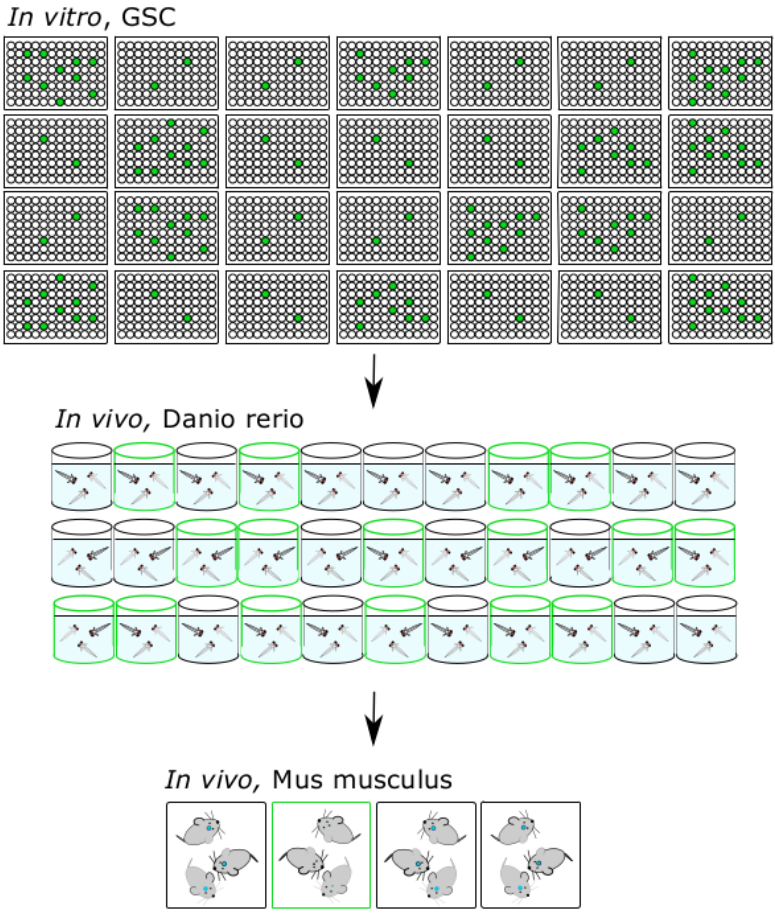

Figure 4: A schematic view on how different models can be used in drug discovery.

Objectives

The overall aim of this thesis was to create representative, stable and well-characterised cell-based models of paediatric HGG. These models allow for the identification of better treatment options as well as an increased understanding of biological and mechanistic processes in tumours.

Specific aims

Paper I

- Establish a robust, well-characterised, *in vitro* system for primary HGG stem cells
- Explore the possibilities of using the system for identification of improved therapy

Paper II

- Establish an orthotopic primary human HGG xenograft mouse model
- Study the clinical relevance of the model

Paper III

- Establish an orthotopic primary human HGG xenograft zebrafish model
- Evaluate the use of the model to identify therapeutic candidates

Methodology

In this thesis, the same patient material was used in the studies; paper I (*in vitro*), paper II (*in vivo*) and paper III (*in vivo*).

Patient material

Regional ethical approval was obtained for the studies (Dnr 604-12). Patient material was donated from children operated for a high-grade brain tumour and signed informed consent was obtained from the parents. Tissue was collected during surgery and was taken to the cell lab for culture preparation within an hour.

Cell culture

In paper I, tumour tissue was mechanically and enzymatically dissociated prior to seeding in culture dishes. Cells were cultured in a serum-free stem cell media containing DMEM-F12 and supplemented with B27, N2 and the growth factor EGF. In this thesis, seven primary paediatric HGG stem cell cultures were established and used; GU-pBT-7 (called BPC-A7 in paper I), GU-pBT-10 (BPC-B0), GU-pBT-15 (BPC-B5), GU-pBT-19 (BPC-B9), GU-pBT-23 (BPC-C3), GU-pBT-28 (BPC-C8) and GU-pBT-58.

Cells were cultured adherently in culture dishes coated with laminin to facilitate cell attachment, or as sphere cultures in low attachment 96-well plates without laminin. Adherent cells were kept in cell culture for up to 30 passages.

To enrich for stem cells and maintain proliferating and non-differentiated cell cultures, EGF was added to the media and the cells were cultured adherently on laminin. A previous study showed that laminin helps

preserve stem cell features in the presence of EGF and in the absence of fibroblast growth factor (FGF) [153]. The study showed that culturing cells on gelatin with removal of both growth factors resulted in astrocyte differentiation and failure of cell culture establishment. Even with the addition of EGF, cells stopped proliferating and died. However, replacement of gelatin with laminin, in the presence of only EGF, resulted in viable and proliferating cells. This study concluded that EGF allows for neural stem cell cultures and self-renewal of these cells. Other studies by Pollard et al and Sun et al also showed the requirement of EGF in neural stem cell culturing [154, 155].

Immunocytochemistry and immunohistochemistry

When visualising proteins or antigens in cells or tissues, immunocyto- and immunohistochemistry are commonly used. These two methods are based on the same antigen-antibody interaction technique. The method was first introduced in the 1940s by Albert H Coons, who studied the possibility to detect antibodies by using optical tracing [156].

Simplified, the refined methods are based on a primary antibody binding to the antigen of interest, which is then detected by a secondary antibody with a detection probe. Commonly used detection probes are horseradish peroxidase (HRP) or fluorochromes. HRP catalyses the cleavage of the chromogen DAB (3,3′-Diaminobenzidine) which in turn is visualised under a light microscope. The HRP can be used as a detection probe in numerous ways. A fluorochrome does not need any additional reactions and can be directly visualised under a fluorescent microscope.

It is important to evaluate the specificity of the antigen-antibody interaction as antibodies can bind un-specifically, thus creating background, or bind to closely related antigens. In addition, immunohistochemistry requires blocking of the endogenous HRP that is present in tissues.

In paper I, stem cell specific antibodies against NESTIN, SOX2, OLIG2 and VIMENTIN were detected by a fluorescent conjugated secondary antibody. Moreover, differentiation of the CSCs was demonstrated using antibodies against the neuronal marker MAP2 and the astrocyte marker Glial fibrillary acidic protein (GFAP). To measure proliferation rate, 5-ethynyl-2-deoxyuridine (EdU) was added to the cell culture, to be incorporated into the genome upon mitosis, 24 hours prior to fixation and detected with a direct fluorescent primary antibody.

In paper I, II and III, immunohistochemistry was performed by staining for human NESTIN, to demonstrate the presence of human GSCs in the mice and fish brains. In these studies, a HRP conjugated secondary antibody was used and the intensity was amplified with an avidin-biotin complex. In addition, the mice brains were stained with the proliferation marker Ki-67 using the same secondary antibody complex.

PDOX mouse model

Prior to transplantation, adherent cells in the exponential phase were enzymatically detached, counted and re-suspended in PBS, and kept on ice until transplantation.

Immunocompromised NOG mice, 6-8 weeks old females, were used for the mouse xenograft model in paper I and II. The mice were anesthetised and placed in a stereotactic frame. The top of the head was shaved and the scull was visualised using a scalpel. To pass through the scull, a hole was drilled approximately 1mm rostral and 2mm lateral (right) to bregma using a hand drill. 100 000 cells in 1µl were injected at a depth of 2.5 mm into the putamen on the right side of the brain [157].

Mice were monitored and weighed continuously until they reached the endpoint when they were euthanized due to weight-loss or physical

disabilities. Brains were removed and fixed in 4% PFA before paraffin-embedding and stored at room temperature until sectioning using a microtome when 5µm sections were mounted on super-frost glass.

In paper I, three GSC lines were used in the PDOX model. In paper II, these three PDOX models were further evaluated together with three new PDOX models.

DNA methylation analysis

For the methylation studies in paper I and II, DNA from tumours and CSC cultures and xenograft mouse tumours, was isolated. To detect methylated cytosines in the samples, bisulfite modification was used, which converts all unmethylated cytosines to uracils (U) [158]. The modified DNA was then amplified by PCR, where the U is amplified as a T.

Methylation values of 450 000 or 850 000 CpG sites, distributed over gene-populated areas of the genome, was measured with the Infinium HumanMethylation450 BeadChip and Infinium MethylationEPIC BeadChip. The array is constructed by a two-coloured system emitting different colours depending on the methylation status of the original DNA strand. The methylation data was analysed using the programming software R (http://www.R-project.org/) with the R-package ChAMP [159]. The relationship between methylated and unmethylated sites is calculated as:

$$\beta = \frac{\max(y_{meth}, 0)}{\max(y_{un-meth}, 0) + \max(y_{meth}, 0) + \alpha}$$

where y_{meth} and $y_{un\text{-}meth}$ are emission intensities and α is set to 100 [160]. The β value ranges between 0 and 1 where β = 0 is completely unmethylated and β = 1 is completely methylated. Clustering analysis and identification of differentially methylated positions (DMP) were

performed using the methylation values. CNA were inferred from the methylation data using the conumee R package [161].

PDOX zebrafish model

Prior to transplantation, adherent cells were evaluated as in paper II. Cells were labelled with CellBrite Cytoplasmic Membrane Dye, a fluorescent membrane dye that enables detection of cells.

In paper III, zebrafish AB wild-type eggs were collected at 0 days post fertilisation (dpf) and sorted based on quality. Embryos were maintained in embryo media (EM) and kept at 28.5°C. At 24 hours post fertilisation, eggs were placed in EM containing 0.003 % w/v 1-phenyl 2-thiourea (PTU) and kept in PTU throughout the experiment to prevent pigmentation.

Xenograft transplantation was performed at 2 dpf and unhatched larvae were dechorionised using forceps. Larvae were anesthetised and embedded in low-melting agarose to maintain a fixed position during the procedure. Cells were loaded into a glass capillary, which was attached to a Narishige MN-153 micromanipulator, and connected to an air-driven Picospritzer microinjector. Approximately 100 cells in a volume of 1 nl were injected by inserting the needle into the forebrain ventricles. Larvae were removed from the agarose after the injection, placed in ringer solution containing PTU and kept in well-plates at 34.5°C.

Larvae were monitored using confocal imaging at 1 day post injection (dpi), 5 dpi and 8-9 dpi. Larvae were also fresh frozen in optimal cutting temperature (OCT) compound at 1 dpi and 8-9 dpi for immunohistochemistry and stored at -80°C until sectioning. Brains were sectioned using a cryostat and 8µm sections were mounted on super-frost glass.

Live imaging

A LSA 710 confocal microscope (Zeiss) was used to monitor glioma cells in the larvae brain in paper III. Larvae were anesthetised and embedded in low-melting agarose to keep the animal fixed during imaging. Fluorescent cells were visualised at 644 nm and a Z stack of 2µm of the larvae brain was acquired by the ZEN Zeiss software. The difference in area of tumour cells in the ventricle area was measured at 1 dpi and 5 dpi and the distance that cells had invaded into the brain was measured at 5 dpi using Image J (NIH) [162, 163].

Statistical methods

Pearson product moment correlation (Pearson correlation)

The strength of a linear association between two variables (x and y) can be measured by Pearson correlation (r) as illustrated below. The values of r range from -1 to 1, where $r < 0$ is a negative correlation and $r > 0$ a positive correlation and 0 means no correlation. The closer r is to -1 and 1, the stronger the correlation is.

$$r = \frac{\sum xy}{\sqrt{\sum x^2 \sum y^2}}$$

Kaplan-Meier survival analysis

In paper I and II, the Kaplan-Meier survival analysis was used to describe the percentage of animals alive after a certain time during the experiment [164].

Bliss independence model

In paper III, the effect of two independent drugs added in combination was evaluated in an attempt to increase the treatment effect. Even though the combination of two drugs results in an increased effect compared to the single drugs alone, we have no knowledge about the relation between the

drugs. Drugs can act in synergy or non-synergy, which is explained in the Bliss independence model [165]. The interaction score is calculated by subtracting the expected effect from the observed effect. The observed effect (D_{AB}) is the result shown in the experiment while the expected effect (D_AD_B) is the product of the observed effect from the single drugs.
Interaction score ($\mathcal{E}$) is calculated:

$\mathcal{E} = D_{AB} - D_AD_B$

$\mathcal{E} < 0$ synergy

$\mathcal{E} > 0$ non-synergy

Results and discussion

Paper I: Stem cell cultures derived from pediatric brain tumors accurately model the originating tumors

HGG is molecularly not the same in adults and children, and available GSC cultures have been established from adults only. In this study, we therefore assessed the possibility to culture primary high-grade GSCs, from children, *in vitro*. An illustration is presented in Figure 5.

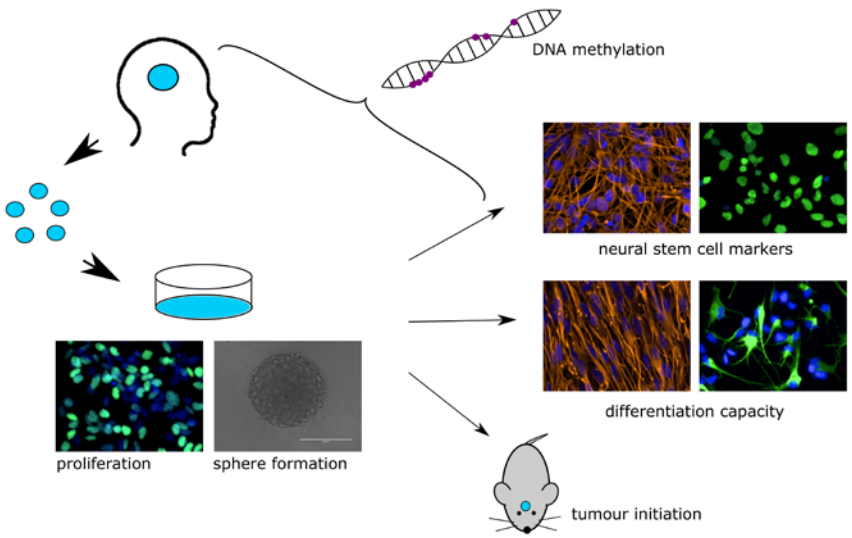

Figure 5: Illustration of the GSC culture *in vitro* model. Proliferating cells are visualised in green (left). Neural stem cells markers; NESTIN (orange) and SOX2 (green). Differentiation capacity; demonstrated with expression of the astrocytic marker GFAP (orange) and the neuronal marker MAP2 (green).

Six primary GSC lines were established. The cells were cultured adherently in serum-free neural stem cell media and was maintained for 30

passages. Genomic and epigenomic analyses of the primary cell cultures showed that the cells were stable during culturing and thus represented a robust model. The cells were positive for stem cell markers and responded to differentiation cues. Three out of three tested primary cell lines initiated tumour growth when orthotopically transplanted into mice.

Tumour samples from the six patients in this study, were diagnosed histologically and by DNA methylation-based classification using the MethPed algorithm [22]. Tumours were diagnosed as GBM, PNET and ATRT by histological diagnosis. In contrast, MethPed classified all samples as GBM. Additional molecular analyses and a second histological review performed by a senior pathologist confirmed the GBM diagnose for all samples.

The tumour cells proliferated at a steady rate during the 30 passages they were kept in culture (rate dependent on cell line), and they were positive for the neural stem cell markers NESTIN, SOX2, OLIG2 and VIMENTIN. When treated with differentiation agents, cells responded by a decrease in proliferation, loss of neural stem cell markers and increase in astrocyte and neuronal cell markers.

To evaluate the tumour-initiating capacity, three of the primary cell lines were orthotopically injected into NOG mice, with a tumour take-rate close to 100% (14/15 injected mice developed tumours). To further prove tumour-initiating capacity of the GSCs, one primary cell line was also orthotopically injected into zebrafish larvae, showing invasive tumour growth after only a few days. To emphasise the importance of using primary cell lines and the fact that non-primary cell lines lose their tumour characteristics, the adult GBM cell line U87 was injected into the zebrafish larval brain. U87 did not show the characteristic invasive growth pattern of GBM, highlighting the lack of relevance of such a cell line (Figure 6).

Thus, the relevance of the cells used in studies should be considered to enable accurate conclusions to be drawn.

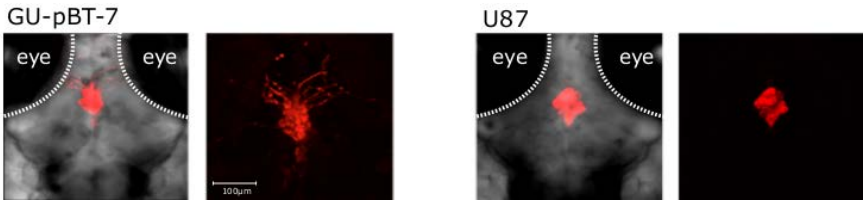

Figure 6: The invasive growth pattern of the primary cell line GU-pBT-7 compared to the non-primary GBM cell line U87 (both labeled with RFP) in larval zebrafish.

Genetic aberrations in tumours are common, although paediatric GBM generally have less genomic instability than adult GBM. CNA inferred from methylation data [166] from tumour tissue in our study showed common aberrations such as *EGFR* amplification and deletions in *RB1*, cyclin-dependent kinase inhibitor 2A/B (*CDKN2A/B*) and phosphatase and tensin homolog (*PTEN*). CNA analysis of the tumours and the corresponding GSC lines showed similar profiles.

To evaluate the stability of the DNA methylation in this *in vitro* model, the methylation pattern of the originating tumour and GSCs from the primary cell cultures were compared. In hierarchical cluster analysis, the GSCs grouped together with its originating tumour and separate from the other cell lines. Further, examining the DMP identified that less than 4% of the studied CpG sites changed methylation state, strengthening the relevance of this *in vitro* model for DNA methylation studies.

Cell lines can alter molecular and physical properties after serial passages in culture due to selection of cells. Sub-clones, or tumour-specific aberrations, can be lost, thereby making the cell line irrelevant for the purpose. Continuous evaluations of cell lines should therefore be performed to ensure that specific tumour characteristics are not lost over

time. In this study, samples were therefore taken from different passages to evaluate the molecular stability and robustness of the cell culture, which showed a close relationship between low, middle and high passage cells.

The use of primary cell cultures instead of non-primary cell lines in cancer research have many advantages. Cell lines that have been cultured for several years might not resemble the original sample. A few years ago, it was shown that the commonly used adult GBM line, U87, did not show similarities with the originating tissue sample and early-cultured cells [167]. This highlights the need for thorough validations of cell lines that are used as basic material in many research projects. The use of primary cell lines reflects tumour properties and specific characteristics of the tumours and add the clinical relevance enabling personalised treatment designs. For example, drug screening in a xenograft zebrafish model with a fast disease course could guide patient treatment.

Moreover, depending on the aim of the study, the experimental set up should promote correct culture conditions. Many GBM tumours exhibit *EGFR* amplification, but this is lost when culturing cells in media supplemented with EGF [168]. For experiments when it is important that the *EGFR* amplification is retained, optimised culture conditions should be used. In this study, we showed that cells cultured without EGF but with the supplement of another growth factor (FGF), retained the *EGFR* amplification after several passages.

The tumour-initiating capacity of CSCs is a fundamental characteristic where tumour formation is a proof of stem cell capacity. The growth pattern and specific morphology of the xenograft tumours are of utmost importance to show clinical relevance. In previous studies, primary CSC lines from paediatric high-grade brain tumours have been described [119]. Tumour-initiating capacity from one of the cell lines, a medulloblastoma group 3, proved the CSC characteristics of that culture in a later publication [169].

An advantage of culturing cells adherently in 2D cultures is that it facilitates the analysis of morphological changes. In addition, cell proliferation and presence of specific antigens can easily be evaluated. The limitations of a 2D cell culture is obviously the lack of the 3D environment that cells are part of in the body. The cell-to-cell communication in a heterogeneous tumour in the body could be mimicked by co-culturing cells either in 2D or 3D to better resemble the natural habitat. Further, culture models that mimic the natural habitat even closer have recently been published by Marques-Torrejon et al who cultured GSCs in sections of mouse brain tissue [170]. This study showed that sections of mouse brain could survive in serum-free media for weeks. GSCs can be injected in specific areas studying growth pattern and environmental influences in different parts of the brain. The model is an interesting addition with great potential as a co-culture system between the *in vitro* and *in vivo* setting.

In this study, we presented a robust and representative adherent serum-free cell culture model established from six paediatric primary high-grade brain tumours. The model is well-characterised showing CSC properties and display genomic and epigenomic stability, with profiles resembling the originating tumours.

Paper II: Cell line-based xenograft mouse model of paediatric glioma stem cells mirrors the clinical course of the patient

Prior to this study, no cell line-based paediatric glioma xenograft model had been described. In paper II, tumour-initiating capacity of primary GSC lines were assessed and a paediatric GSC-based PDOX model in mice was established. Six primary GSC lines were transplanted into NOG mice and the survival time of the mice was studied. The morphology and growth pattern of the tumour cells in the xenograft tumours was assessed. The diagnosis of the xenograft tumours were based on histological diagnosis and molecular diagnosis. Further, genetic and epigenetic stability of the

xenograft tumours in comparison to the GSC lines and the originating tumours were evaluated. The model is illustrated in Figure 7.

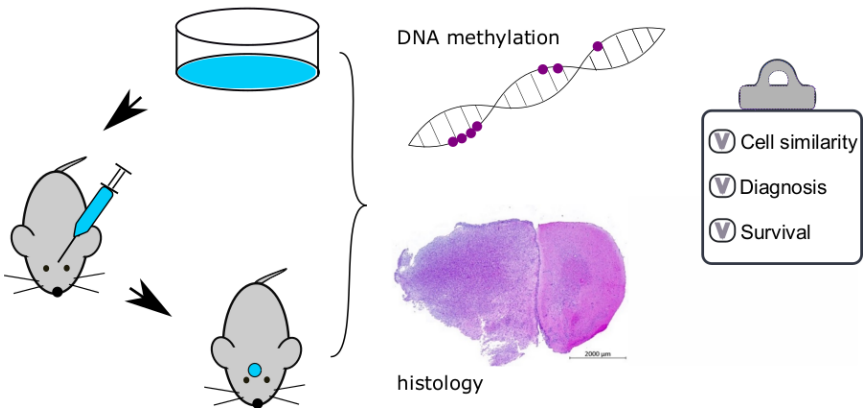

Figure 7: Illustration of the orthotopic HGG mouse xenograft model.

In this study, six GSC lines were orthotopically transplanted into immunosuppressed mice. Take-rate for each GSC line was close to 100% (29/30 mice) and the time-span between deaths were close in each group.

The survival time of mice correlated well with the patient survival time. This is of utmost importance since the GSCs produce a tumour with similar features in the context of growth patterns as the originating patient tumour. A robust and reliable *in vivo* model is important to draw relevant conclusions from experiments.

All xenograft tumours presented GBM characteristics of diffuse infiltration, endothelial proliferation and atypical mitosis. Moreover, xenograft GBM tumours formed Scherer´s secondary structures with perineuronal and leptomeningeal accumulation and perivascular infiltration. The proliferation marker Ki-67 indicated massive tumour growth. Additionally, all xenograft tumours were diagnosed as GBM using the MethPed classifier [22].

Further characterisation of the xenograft tumours showed that genetic aberrations seen in the tumour tissue and GSC lines, were also present in the xenograft tumours. The epigenetic stability was visualised in a hierarchical clustering, which clustered the originating tumour, GSC line and xenograft tumour together based on DNA methylation data.

Establishing xenograft mouse models can be done in different ways. Other PDOX models were based on transplantation of human paediatric tumour tissue into mice [126, 171]. This approach is time consuming and expensive since cell lines have to be established by serial passaging in mice prior to experimental testing. According to the engraftment rates in the Children's Oncology Group biobank, the success of HGG tumour formation was approximately 30% [126]. In another study by Kim et al, it was shown that transplantation of glioma tissue failed to engraft [172]. Depending on how many tumour-initiating cells that are transplanted into each mouse, the tumour growth and survival of the mice will differ. In contrast, cell line-based xenograft models produce a uniform model where all tumours are formed from GSCs. This is an advantage when comparing results from different xenograft experiments and it **reduces** the number of animals needed. This is in stark contrast to the uncertainty of engraftment using tumour tissue, which contributes to unnecessary use of animals. Moreover, Ben-David et al showed that human cells could be influenced by the mouse environment after serial passaging, thus altering important features of human tumour cells [173]. By using cell lines established *in vitro*, the serial passaging of human tumour cells in mice can be avoided.

As with all animal models, there are limitations. The obvious one is that a mouse is not a human. Although the mouse brain contains all the same cell types as in humans, the mouse is still a mouse. The impact of mouse cells on human cells will alter the human GBM cells over time in any model. Moreover, the xenotransplanted mice are immunosuppressed to allow for an allogenic tumour growth, which is a disadvantage. Further, the

experimental time from initiation of a tumour to the completion of the experiments is long and it can take many months to get results from a study.

Even with limitations, our model offers a robust and reliable alternative to previous mouse models, and is to our knowledge the first paediatric primary cell-based model that correlates with patient survival, which strengthens the clinical relevance of the model.

An advantage of cell-based models is that fewer mice are used, since passaging and establishment of primary cell lines are performed in cell culture. With fewer animals used, this model offers a less expensive and less work-demanding model. The model is therefore in agreement with both **reduction** and **replacement**.

Paper III: Invasion of human paediatric high-grade gliomas in zebrafish

In paper III, we explored the use of zebrafish as an additional animal model for paediatric HGG. The previous pilot study in paper I showed promising results that were elaborated in this study. Tumour invasive properties of five GSC lines were studied. As a proof of concept, treatment response to TMZ, VPA and decitabine was evaluated (Figure 8).

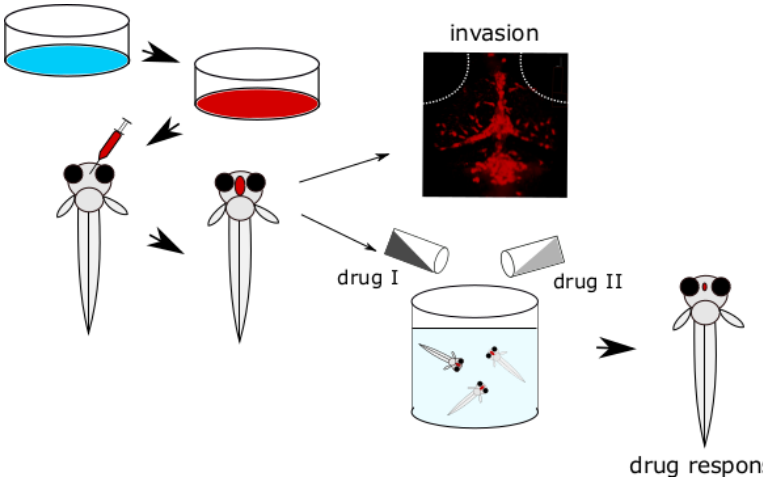

Figure 8: Illustration of the zebrafish model. GSCs are stained red prior to injection using a membrane dye.

Zebrafish have been used as transgenic models, PDX and PDOX models in different types of disease-related research [129, 130, 137, 141, 149]. This animal model offers a feasible, fast, less expensive and experimentally less work-demanding model compared to other commonly used models such as rodents.

In this study, five primary paediatric GSC lines were orthotopically injected into the frontal ventricles of the zebrafish larvae. This resulted in tumour cell invasion in 98% of the cases. Post-injected deaths due to technical injuries were low; only 4/60 animals had to be removed from the study. Compared to a previous model where injections were performed into the blastula of the egg, transplanting tumour cells into the larvae, as done in this study, generated a higher take-rate which allows less animals to be used [143]. Invasion was assessed at 5 dpi by counting the number of tumour cells in the brain tissue and the distance the cells had migrated from the midline of the brain.

As mentioned in paper II, GBM is characterised by diffuse infiltration. Compared to the infiltrative pattern of the GBM cell line U87 in paper I, where infiltration was completely absent, this primary cell line-based model showed infiltrative properties.

One technical difficulty with using zebrafish as an animal model is the difference in temperature that fish and human cells require. The natural laboratory habitat for zebrafish is a water temperature of 28°C while the human cells grow at 37°C. To adjust for the temperature difference, previous studies have been carried out housing the fish at 34°C post injection since zebrafish have been shown to survive a habitat of higher temperatures without developing malformations and disabilities [137, 141, 142]. In addition, in our study, the primary GSC lines continued to proliferate and were viable, *in vitro*, at 34°C, although the proliferation was slower. There is no consensus in the literature regarding which temperature the human tumour cells should be cultured at prior to injection [141, 142]. We therefore compared the two culture conditions (34 and 37°C) prior to injection to study the take-rate and invasive properties. This resulted in no difference between the two temperatures. We thus concluded that human tumour cells do not have to be acclimatised to the lower temperature and can therefore be grown at 37°C prior to injection without affecting the result.

GSCs were visualised in the fish brain with an antibody specific for human NESTIN, a neural stem cell marker. GSCs were present the day after injection and after 8-9 days, demonstrating that human cells survive in the fish brain.

To evaluate the use of the developed models for drug testing, three primary GSC lines were treated, *in vitro*, with TMZ, VPA and decitabine, which resulted in a significant decrease (p-value < 0.05) in number of cells for all treatments. In addition, the combination of TMZ and VPA resulted in an

enhanced treatment response due to a synergy effect between the drugs, calculated with the Bliss-independence model.

The drug treatments were also performed *in vivo* using larvae injected with the GSC culture GU-pBT-19. Drugs were added to the EM in a concentration that allowed for normal development of the larvae. Treatment response was evaluated at 5 dpi and resulted in a decreased number of tumour cells that had invaded the fish brain. This correlated strongly with the results from the *in vitro* study.

The use of primary GSC lines makes it possible to study patient-specific treatment alternatives and propose further treatment within a feasible time to benefit the patient. In paper I, and in previous studies by others, it was shown that the survival time of tumour-injected larvae was approximately 10-30 days [141, 142]. This is an advantage studying personalised treatment strategies as results can be obtained in a timely manner. However, treatment that spans over a longer period could be harder to achieve using this model.

A disadvantage of fish in comparison to mammalian models include the absence of specific organs such as breast, prostate and lungs. Orthotopical xenograft models are consequently not an option for studies in these organs. In addition, studies of immune response in early life are not possible due to the lack of an adaptive immune system.

To summarise, in this study we show that zebrafish is a feasible animal model for primary paediatric HGG. The model enables cell invasion studies and evaluation of treatment potential. This model shows stable tumour initiation and high transplantation take-rate using multiple cell lines.

Conclusion and future perspective

This thesis contributes to cancer research by presenting three pre-clinical models of paediatric HGG. Pre-clinical work is of importance for finding better treatment solutions, avoiding toxic drug effects and evaluating patient-based strategies. Pre-clinical models contribute with results and conclusions that are passed on to the next model with the hopes of reaching human studies and benefit patients. Creating a perfect model is not possible though, as animals are different from humans, but this should not discourage us. The use of primary tumour tissue and primary cell lines provides the possibility to create models reflecting the patient diversity. This is a great advantage over the use of traditional cell lines, as in fact, humans are different from humans too.

To conclude, the *in vitro* model presents serum-free cell lines representative of the originating tumours. The workload is less demanding than using a PDX model to establish a cell line. In contrast to using traditional cell lines, primary cell lines add patient specificity that gives a broader knowledge contributing to the clinical relevance.

The *in vivo* mouse PDOX model introduces the missing piece, the paediatric angle, important and needed. Children are not tiny adults, and should therefore have pre-clinical models based on the right type of material. In this study, the clinical relevance is highlighted in the similarities between the original tumour and the xenograft tumour. The correlation in survival time between patients and mice shows that the model mimic the clinical course of the patient.

The zebrafish PDOX model contributes to the research field by adding a novel and interesting approach to the *in vivo* stage. Although previous models have shown that human brain tumours can grow in fish, we show

that primary cell lines based on paediatric tumours can be used in this model.

Both *in vivo* models presented in this thesis are based on immune incompetent animals. One of the hallmarks of cancer points out the close interaction between cancer cells and immune cells. For this reason, novel models using immunocompetent animals in xenograft studies are under development and should be considered in paediatric studies [174].

Pre-clinical models are of great interest and a necessity in medical research. Different research questions require different applications and models. Every new model can increase our knowledge, which enable us to develop new models that are more precise. Remember; **reduction**, **refinement** and **replacement**.

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